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Altered MicroRNA Profile in Osteoporosis Caused by Impaired WNT Signaling

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related to the application of circulating microRNAs as biomarkers for diagnosis of bone disorders. The other authors have declared that no conflicts of interest exist.

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ABSTRACT

Context: WNT signaling is fundamental to bone health and its aberrant activation leads to skeletal pathologies. A heterozygous missense mutation p.C218G in *WNT1*, a key WNT pathway ligand, leads to severe early-onset and progressive osteoporosis with multiple peripheral and spinal fractures. Despite the severe skeletal manifestations, conventional bone turnover markers are normal in mutation-positive patients.

Objective: This study sought to explore the circulating miRNA pattern in patients with impaired WNT signaling.

Design and Setting: A cross-sectional cohort study at a University Hospital.

Participants: Altogether 12 mutation-positive (median age 39 years, range 11–76 years) and 12 mutation-negative (35 years, range 9–59 years) subjects from two Finnish families with WNT1 osteoporosis due to the heterozygous p.C218G *WNT1* mutation.

Methods and main outcome measure: Serum samples were screened for 192 miRNAs using qPCR. Findings were compared between *WNT1* mutation-positive and mutation-negative subjects.

Results: The pattern of circulating miRNAs was significantly different in the mutation-positive subjects as compared with the mutation-negative subjects with 2 upregulated (miR-18a-3p, miR-223-3p) and 6 downregulated miRNAs (miR-22-3p, miR-31-5p, miR-34a-5p, miR-143-5p miR-423-5p, miR-423-3p). Three of these (miR-22-3p, miR-34a-5p, and miR-31-5p) are known

inhibitors of WNT signaling: miR-22-3p and miR-34a-5p target *WNT1* mRNA and miR-31-5p is predicted to bind to *WNT1* 3'UTR.

Conclusions: The circulating miRNA pattern reflects *WNT1* mutation status. The findings suggest that the *WNT1* mutation disrupts a feed-back regulation between these miRNAs and WNT1, providing new insights into the pathogenesis of WNT-related bone disorders. These miRNAs could offer future potential in diagnosis and treatment of osteoporosis.

Précis

This study reports a unique serum expression pattern of 8 circulating miRNAs in *WNT1* mutation-positive subjects and a novel finding of a communication between WNT1 and miR-31-5p.

INTRODUCTION

Bone health is maintained by precisely balanced bone formation and resorption. In addition to key regulatory pathways and endocrine factors, microRNAs (miRNAs) have recently emerged as integral modulators of bone metabolism (1,2). MiRNAs are short, noncoding RNA fragments that regulate target gene expression by posttranscriptional silencing or repression of protein translation and serve important functions in various biological processes (3). In bone, miRNAs regulate both osteogenesis during fetal development and maintenance of bone health postnatally (3-6). In vitro studies further demonstrate their direct role in regulation of osteoblast and osteoclast development, maturation, and function (7-11). Furthermore, some clinical studies suggest that expression levels of different miRNAs associate with idiopathic and postmenopausal osteoporosis, correlate with bone mineral density (BMD), and differentiate between patient cohorts (12-16). Based on these observations miRNAs have been anticipated to have applications in the diagnosis and treatment of bone diseases, including osteoporosis (12,16).

Canonical WNT/β-catenin pathway is a key regulator of bone metabolism and defective WNT signaling underlies several monogenic skeletal disorders with low or high bone mass, such as osteoporosis-pseudoglioma syndrome, van Buchem disease, and sclerosteosis (18-20). In 2013, our research group identified WNT1 as a major regulator of bone mass as a heterozygous missense mutation p.C218G in *WNT1* was shown to lead to decreased activity of the WNT/β-catenin pathway in bone, resulting in low bone turnover with decreased number of bone cells and impaired bone formation, and consequently low bone mass and skeletal fragility (21). The mutation was first reported in a large Finnish family exhibiting severe, early-onset osteoporosis, multiple peripheral and spinal compression fractures, and subsequent loss in adult height (21,22). Despite the low BMD and significant skeletal pathology, the conventional bone biomarkers currently in clinical use, such

as alkaline phosphatase (P-ALP), the bone formation marker N-terminal propeptide of type I procollagen (PINP), and the bone resorption marker type I collagen cross-linked N-telopeptide (INTP), do not differ between *WNT1* mutation-positive and mutation-negative individuals (21,22).

Recent in vitro studies show that specific miRNAs regulate WNT signaling by binding to the pathway's key components and inhibitors: miR-152-3p and miR-335 to Dickkopf-1, miR-30e-5p to low-density lipoprotein-receptor 6, and miR-27a-3p, miR-142, and miR-135a to adenomatous polyposis coli (23-28). Data on miRNA expression and circulating miRNA levels in genetic bone diseases are, however, still scarce (29). In patients with altered WNT signaling, miRNA profile, miRNAs' potential role in disease pathogenesis, clinical diagnostics and follow-up remain unknown and previously unexplored. To gain more insight to the clinical relevance of miRNAs in inherited bone diseases with defective WNT signaling, we assessed miRNA profiles in subjects with a heterozygous *WNT1* mutation and their mutation-negative family members.

SUBJECTS AND METHODS

Subjects. We have previously identified two large Finnish families with autosomal dominant WNT1 osteoporosis (21,22). The first family (Family A) was identified in 2013 as a novel heterozygous missense mutation p.C218G in *WNT1* was identified by linkage analysis and targeted sequencing as the cause for severe early-onset osteoporosis in a family with 10 affected family members (21). We have since offered genetic screening to all first-degree relatives at risk and, subsequently, 21 additional mutation-positive family members have thus far been identified (Family A). A second family (Family B) was found to harbor the same exact point mutation as the whole WNT1 gene was Sanger sequenced for > 250 other Finnish index patients with osteoporosis. In this

family, 4 subjects have so far been found to harbor the same mutation. Hence, to date, altogether 25 mutation-positive subjects with the heterozygous p.C218G have been identified in Finland.

For the study, we offered all mutation-positive subjects from Family A and Family B the opportunity to participate in a study concerning miRNA studies in search for new bone biomarkers associated with the heterozygous *WNT1* mutation. A control group with similar age and sex distribution was formed by offering the mutation-negative subjects in Family A (n = 29) and Family B (n = 3) also the opportunity to participate. Altogether 17 mutation-positive and 18 mutation-negative individuals consented.

All participants filled out a questionnaire with the following information: other diagnosed diseases, all previous fractures, all previous surgeries, previous bisphosphonate or other osteoporosis medication, other medication, and calcium and vitamin D supplementation. Based on these anamnestic data, we aimed to exclude subjects with fractures or orthopedic surgeries in the past 12 months, or possibly confounding illnesses or medications.

Genetic evaluations. We screened all participating study subjects for the heterozygous missense mutation c.652T>G (p.C218G) in exon 4 of *WNT1* (NCBI Reference Sequence NM_005430.3). We performed genetic validations on DNA extracted from peripheral blood as previously described (22).

Serum samples. We collected all serum samples during the spring season (January-April) in 2017 to avoid bias from variable sunlight exposure. All samples were taken after a 12-hour fast, in the morning between 0800 h and 0900 h. Venous blood was first collected into normal serum tubes and left to stand in room temperature for 30-60 minutes to allow clotting. The tubes were then

centrifuged at 2500 x g for 10 minutes in room temperature and the supernatant was transferred to 1.5 ml tubes in 250 µl aliquots. The serum was immediately stored at -80°C until analysis.

Blood biochemistry. We assessed blood biochemical values from separate peripheral blood samples for serum calcium, phosphate, alkaline phosphatase (ALP), 25-hydroxyvitamin D (S-D-25; assessed with immunochemiluminometry), and 1,25-dihydroxyvitamin D (S-D-1,25; assessed with immunochemiluminometry); results were compared with laboratory's reference values. Serum parathyroid hormone (PTH) and collagen type 1 cross-linked C-telopeptide (CTX-1; a bone resorption marker) were analyzed with an IDS-iSYS fully automated immunoassay system (Immunodiagnostic Systems, Ltd., Bolton, UK) with chemiluminescence detection and compared with manufacturer's reference values. Serum intact Fibroblast growth factor 23 (FGF23) was assessed by manual enzyme-linked immunosorbent assay (ELISA; Immutopics International, San Clemente, California, USA; and Kainos Laboratories, Tokyo, Japan); the manufacturer's reference values were used.

miRNA analysis. We isolated RNA from the serum samples using the miRNeasy Mini Kit (Qiagen, Germany). Serum samples were thawed on ice and centrifuged at 12,000g for 5 minutes to remove any cellular debris. For each sample, 200 µL of serum was mixed with 1000 µL Qiazol and 1µL synthetic Spike-Ins (Exiqon, Denmark). After a 10-minute incubation at room temperature, 200 µL chloroform were added to the lysates followed by cooled centrifugation at 12,000g for 15 minutes at 4°C. Precisely 650 µL of the upper aqueous phase were mixed with 7 µL glycogen (50mg/mL) to enhance precipitation. Samples were transferred to a miRNeasy mini column, and RNA was precipitated with 750 µL ethanol followed by washing with RPE and RWT buffer. RNA was eluted in 30 µL nuclease free water and stored at -80°C until further analysis.

Starting from total RNA samples, we synthesized cDNA using the Universal cDNA Synthesis Kit II (Exiqon, Denmark). We chose reaction conditions according to recommendations by the manufacturer. The protocol was modified in that 4µL of total RNA was used per 10 µl reverse transcription (RT) reaction. To monitor RT efficiency and presence of impurities with inhibitory activity, a synthetic RNA spike-in (cel-miR-39-3p) was added to the RT reaction. PCR amplification was performed in a 384-well plate format using custom Pick&Mix plates (Exiqon, Denmark) in a Roche LC480 II instrument (Roche, Germany) and EXiLENT SYBR Green mastermix (Exiqon, Denmark) with the following settings: 95°C for 10 min, 45 cycles of 95°C for 10 s and 60°C for 60 s, followed by melting curve analysis. To calculate the cycle of quantification values (Cq-values), the second derivative method was used. Cq-values were normalized to the mean Cq-value in each sample (global mean normalization) by subtracting the individual miRNA Cq-value from the Cq average calculated for that sample. Experimental design, platform description, and raw as well as normalized data have been submitted to Gene Expression Omnibus according to MIQE guidelines (accession number GSE103473).

Statistical analysis. We used ClustVis to perform exploratory data analysis (30). PCA and hierarchical clustering analyses were performed using the default settings, i.e. singular value decomposition with imputation, correlation as the distance metric, and average distance for clustering. Group-wise differential expression analysis was performed based on global mean normalized delta Cq-values under the assumption of normal distribution, which was assessed visually, using two-sided t-tests. Receiver operator characteristic (ROC) analysis was performed in order to evaluate the power of selected circulating miRNAs to distinguish *WNT1* mutation-positive subjects from mutation-negative subjects. ROC analysis was performed using MedCalc Statistical Software version 18 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2018) to compute area-under-the-curve (AUC) values.

Study approval. All genetic and clinical studies were approved by the Research Ethics Board of Helsinki University Hospital. All subjects gave a written informed consent prior to participation in the study.

RESULTS

Clinical and biochemical characteristics

We recruited study subjects from two families with autosomal dominant WNT1 osteoporosis. Based on pedigree analysis, we selected 12 mutation-positive (hereafter referred to as MP) and 12 mutation-negative (hereafter MN) family members for the analyses (Figure 1, Table 1), aiming at comparable age- and sex distribution in the MP and MN groups. The selected subjects were negative for all four (22 subjects) or for three (2 subjects) of the following exclusion criteria: 1) a fracture in the past 12 months, 2) bisphosphonate or other osteoporosis medication in the past 12 months, 3) hypothyroidism, and 4) use of aspirin or other possibly confounding medication. The MP cohort thus represented approximately half of all Finnish subjects identified with a *WNT1* mutation.

The MP group comprised 7 females and 5 males (age range 11–76 years, median 39 years) and the MN group 5 females and 7 males (age range 9–59 years, median 34,5 years) (Table 1, Figure 1). The MP subjects had DXA-measured BMD values ranging from normal to osteoporosis (Z-score <-2.5), a history of multiple fractures and other skeletal manifestations, but none had had fractures or orthopedic surgeries in the past 12 months and none had other confounding illnesses (Supplemental Table 1). Regarding medications, 3 MP subjects had received previous osteoporosis medication more than 12 months prior to the study, while 1 subject (MP-4) had received the most recent dose of low-dose pamidronate treatment (1 mg/kg once every 4 months) 3 months prior to the study.

Another male subject (MP-13) used aspirin. Serum 25-OH-vitamin D concentration in the MP group was slightly higher (90 nmol/L vs. 68 nmol/L) than in the MN group as the MP subjects also received, on average, a higher vitamin D supplementation (53 ug/day vs. 9 ug/day). Other parameters of calcium homeostasis and bone turnover, including S-D-1,25, PTH, and CTX-1, and intact FGF23 were similar in MP and MN groups (Table 1, Supplemental Figure 1). In the MN group, 7/12 subjects had previously been assessed with DXA or spinal MRI with no signs of osteoporosis or spinal compression fractures (Supplemental Table 1) (21,31).

Quality control of miRNA quantitation

We used a previously described analytical workflow to screen 192 distinct microRNA species and controls in the 12 MP and 12 MN subjects (14). Spike-in controls added prior to RNA extraction, reverse transcription and qPCR amplification were used to assess the technical variance of the workflow (Supplemental Figure 2) and identify potential outliers. We observed low technical variance in spike-in controls (11–23% coefficient of variation (CV)). To exclude a potential bias in our data due to hemolysis, we used the method reported by Blondal et al. of calculating a hemolysis index based on the ratio of miR-451a versus miR-23a-3p (32,33). None of the samples exhibited a ratio of >7, which would indicate hemolysis. The overall sensitivity of the analysis was very good. Only in 6 out of 24 samples missing values for low abundant miRNAs were observed. The maximum number of missing miRNA values observed in any sample was 2 out of 192 analyzed miRNAs (Supplemental Data 1).

Exploratory data analysis

We performed unsupervised exploratory data analyses in the form of principal component analysis (PCA) and hierarchical clustering to assess the overall impact of the MP subjects' *WNT1* mutation on circulating miRNA patterns in comparison to the MN subjects. Using data from the 50 most

variable miRNAs (sorted by CV%), we observed that the overall circulating miRNA levels were not significantly determined by *WNT1* mutation status, sex, or subfamily division (Figure 2A, Supplemental Figure 3). However, group-wise comparison of circulating miRNA levels between MP and MN subjects identified a balanced number of up- and down-regulated miRNAs (Figure 2B). In total, this screening, after applying a low-stringent p-value cut-off of <0.1 to reduce the number of false-negatives (Table 2), identified 16 putative miRNAs which together enabled good discrimination between MP and MN subjects (Figure 3A).

Two of these miRNAs (miR-18a-3p, miR-223-3p) were significantly upregulated ($p<0.043$), while 5 miRNAs (miR-22-3p, miR-34a-5p, miR-423-5p, miR-423-3p, miR-143-5p) were significantly down-regulated ($p<0.045$) in MP subjects (Figure 3B–I). Two miRNAs, miR-22-3p and miR-34a-5p, were found to exhibit good classification performance with AUC-values reaching 0.896 and 0.868, respectively. Correlation between the significantly regulated miRNAs was observed at varying, moderate degree, suggesting that some single miRNAs might contain information derived from different phenotypic characteristics caused by the *WNT1* mutation (Supplemental Table 2). Ongoing low-dose bisphosphonate treatment in one mutation-positive female (MP-4) had no effect on miRNA values (Supplemental Figure 4)

In silico prediction of miRNA targets

We used Targetscan release 7.1 to identify predicted miRNA binding sites in the 3'UTR of human WNT1 gene (34). The tool reported putative binding sites for three miRNAs – miR-22-3p, miR-34a-5p, and miR-31-5p – which we found to be down-regulated in serum of the MP subjects. For miR-34a-5p and miR-22-3p, binding to and regulation of *WNT1* has been experimentally validated (35-38). For miR-31-5p, experimental confirmation of a direct interaction with *WNT1* has not been

previously reported, while its interference with the WNT signaling ligand Frizzled 3 (FRZ3) has been observed (9).

DISCUSSION

This study is the first to report on miRNA profiles in subjects with a monogenic bone disease due to defective WNT signaling. All our mutation-positive subjects harbored a heterozygous missense mutation p.C218G in *WNT1*, which has been previously shown to lead to decreased WNT signaling, low bone formation, low BMD and fractures (21,22) while the mutation-negative subjects have normal BMD and no fractures (21,31). We screened a custom-designed panel of 192 miRNAs and compared the results between 12 mutation-positive and 12 mutation-negative subjects. Our results show that a unique profile of eight miRNAs differentiates between mutation-positive and mutation-negative subjects and that three of these miRNAs – miR-22-3p, miR-34a-5p, and miR-31-5p – are down-regulated in serum in mutation-positive subjects ($p=0.001$, $p=0.003$, and $p=0.053$, respectively). All three miRNAs are known inhibitors of WNT signaling and miR-22-3p and miR-34a-5p have been shown to target *WNT1* specifically (35-38). To the best of our knowledge, no previously published data have reported direct association between miR-31-5p and *WNT1*.

Recent research has found circulating miRNAs as promising new markers in various diseases, including malignancies, as many conventional biomarkers have shown limitations in diagnostics and in evaluating treatment outcomes (29). Regarding bone health and disease, current conventional metabolic bone markers are inadequate in reflecting bone health status, predicting future fracture risk, or monitoring treatment efficacy (12,39,40). Circulating miRNAs show promise as future bone markers, as specific miRNAs that discriminate e.g. patients with manifest osteoporosis (14,16,41), have also been shown to influence bone metabolism in vitro (1,13,14,24,42-43) and in vivo (41,16).

This suggests that circulating miRNA-based biomarkers might have causal links to the disease phenotype, as miRNAs packaged in extracellular vesicles or in protein particles can be taken up by recipient cells in auto-, para-, or even endocrine manner (45).

Of the 8 miRNAs identified in our study as discriminating between MP and MN subjects, 7 have been reported to influence bone metabolism (Table 3). Interestingly, 3 of the downregulated miRNAs in the MP subjects have been confirmed or predicted to directly target WNT signaling and, specifically, *WNT1* mRNA. For one, miR-22-3p negatively regulates osteogenesis and osteoblastogenesis through WNT signaling by targeting the coding region and suppressing the expression of β-catenin, which inhibits formation of calcium nodules during osteoblast differentiation (10). miR-22-3p also targets and decreases the levels of Tcf7 and Ep300, which are key transcriptional proteins for WNT pathway's target gene expression (46). Further, the 3'UTR of *WNT1* contains a binding site for miR-22-3p and miR-22-3p directly targets WNT1 (35). Secondly, miR-34a-5p interacts with WNT pathways components (47) and also, directly with *WNT1* to regulate its mRNA expression and posttranscriptional translation (36-38). Lastly, Weilner et al. showed that miR-31-5p interacts with a WNT pathway component Frizzled-3 (FZD3) and suppresses WNT signaling (9), while Xi et al. showed miR-31-5p to target WNT pathway antagonists Dkk-1 and DACT3 in lung cancer cells (48). However, unlike miR-22-3p and miR-34a-5p, no previous findings of miR-31-5p directly targeting *WNT1* have been reported. Intriguingly, our findings suggest that decreased WNT signaling due to mutated WNT1 leads to downregulation of the miRNAs that exhibit suppressive action on the WNT pathway. This could be regarded as an attempt to normalize WNT signaling in a situation where WNT1-related WNT signaling is impaired. The molecular and genetic feedback mechanisms, including gene regulation by miRNAs, governing balanced bone metabolism are inadequately understood.

To the best of our knowledge, miR-423-3p, which was significantly downregulated in our mutation-positive subjects, has not been previously linked to bone metabolism or WNT signaling. Wang et al. previously reported that miR-423-3p expression changed with age in mouse bone marrow mesenchymal stem cells' microvesicles but no evidence for its effect on osteogenesis was shown (49). Also, other studies have reported that miR-423-3p is linked to myocardial tissue and heart diseases (50,51), but no specific heart phenotype or increased prevalence of myocardial diseases was observed in our cohort or in other *WNT1* mutation-positive subjects (21,22). Therefore, this is the first study to suggest an association between miR-423-3p and WNT pathway.

Prior or on-going osteoporosis medication could potentially alter an individual's miRNA expression, although the mechanisms and exact consequences are still unclear (52). Our cohort included altogether 3 subjects with previous osteoporosis medication and 1 subject with on-going low-dose pamidronate treatment at the time of the study. Separate analysis showed that bisphosphonate treatment had no impact on the expression of the 8 specific miRNAs in our cohort and therefore the observed differences between MP and MN groups are unlikely to be related to the patients' medical therapy. We have previously shown that conventional bisphosphonates have little to no effect on the BMD status in *WNT1* mutation-positive patients (22).

The therapeutic potential in miRNAs has been contemplated and experimentally tested in previous studies; Krzeszinski et al. could attenuate postmenopausal osteoporosis in ovariectomized mice with administration of systemic miR-34 (53), Wang et al. showed anabolic potential of anti-miR-214 in stimulating bone formation in mice (54), and Wang et al. demonstrated protection against glucocorticoid-induced bone loss with miR-29a in rats (42). In a wider context, miRNAs are used clinically in cancer treatment as replacement therapies, to sensitize tumors to chemotherapy, and to treat drug-resistant malignancies (42). Whether these miRNAs up- or down-regulated in our study

could have therapeutic potential – as drug targets or exogenously administered medications – in WNT1 or other forms of osteoporosis should be explored in future experimental settings.

Our study provides novel data on miRNA expression levels in WNT-related bone disease. The study could have been strengthened by larger cohort sizes and with only subjects with no confounding factors, such as osteoporosis medication. We did however exclude subjects with recent fractures and showed that subjects with previous or ongoing osteoporosis medication did not differ in their miRNA pattern from the others. Further, in order to minimize the impact of other confounding genetic factors, we recruited the MN subjects from the same families as MP subjects. We also consider the pre- and post-menopausal status in female subjects as a possible confounding factor. However, the two cohorts were very similar in age and gender distribution, alleviating the possible bias. Furthermore, there is no overlap with the miRNAs reported in this study and those previously reported to have alternate expression due to changes in estradiol concentration in Finnish subjects (55). The small sample size may have prevented us from observing some important differences between the groups, leading to a high false-negative rate. We tried to account for this by allowing relatively high type-I error ($p<0.1$) for initial selection. An independent cohort of *WNT1* MP/MN subjects would be required to validate miR-22, miR-34a and miR-31 findings. However, due to the scarcity of subjects with confirmed WNT1 or other WNT pathway-related osteoporosis, such a study was not possible. Despite these limitations and acknowledging the worldwide rarity of WNT1 osteoporosis, *WNT1* mutation-positive individuals, and the overall scarcity of miRNA data in monogenic bone disorders, we consider our study setting and findings valid and valuable.

We conclude that a unique miRNA profile is observed in *WNT1* mutation-positive individuals compared to healthy individuals. These observations provide valuable information about the molecular pathways involved in WNT1 osteoporosis and the effect of aberrant WNT signaling on

miRNA expression. Our data also provides a novel finding of an association between *WNT1* and miR-31-5p and miR-423-3p expression. The specific miRNAs highlighted in this study could serve as circulating metabolic bone markers in WNT1 osteoporosis to evaluate bone health, fracture healing, and treatment efficacy in affected individuals. Future studies are encouraged to further explore these specific miRNAs in other WNT pathway –related skeletal diseases, their response to anti-osteoporosis treatment, and their potential utilization in the development of osteoporosis treatment.

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AUTHOR CONTRIBUTIONS

Study design: REM, MH, RN, JG and OM. Study conduct: REM, MH, JG, and OM. Data collection: REM, MH, RN, SK and JG. Data analysis: REM, MH and JG. Drafting manuscript: REM, MH, JG. Revising manuscript content: all authors. Approving final version of manuscript: all authors. REM, MH, JG and OM take responsibility for the integrity of the data.

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FIGURE LEGENDS

Figure 1. Pedigrees of the families with a heterozygous p.C218G *WNT1* mutation. Squares represent males, circles females, black symbols mutation-positive family members, white symbols mutation-negative family members, grey symbols mutation-negative healthy controls, and slashes deceased family members. Subjects included in this study are indicated with codes, MP = mutation-positive, MN = mutation-negative. The pedigree has been altered to ensure anonymity.

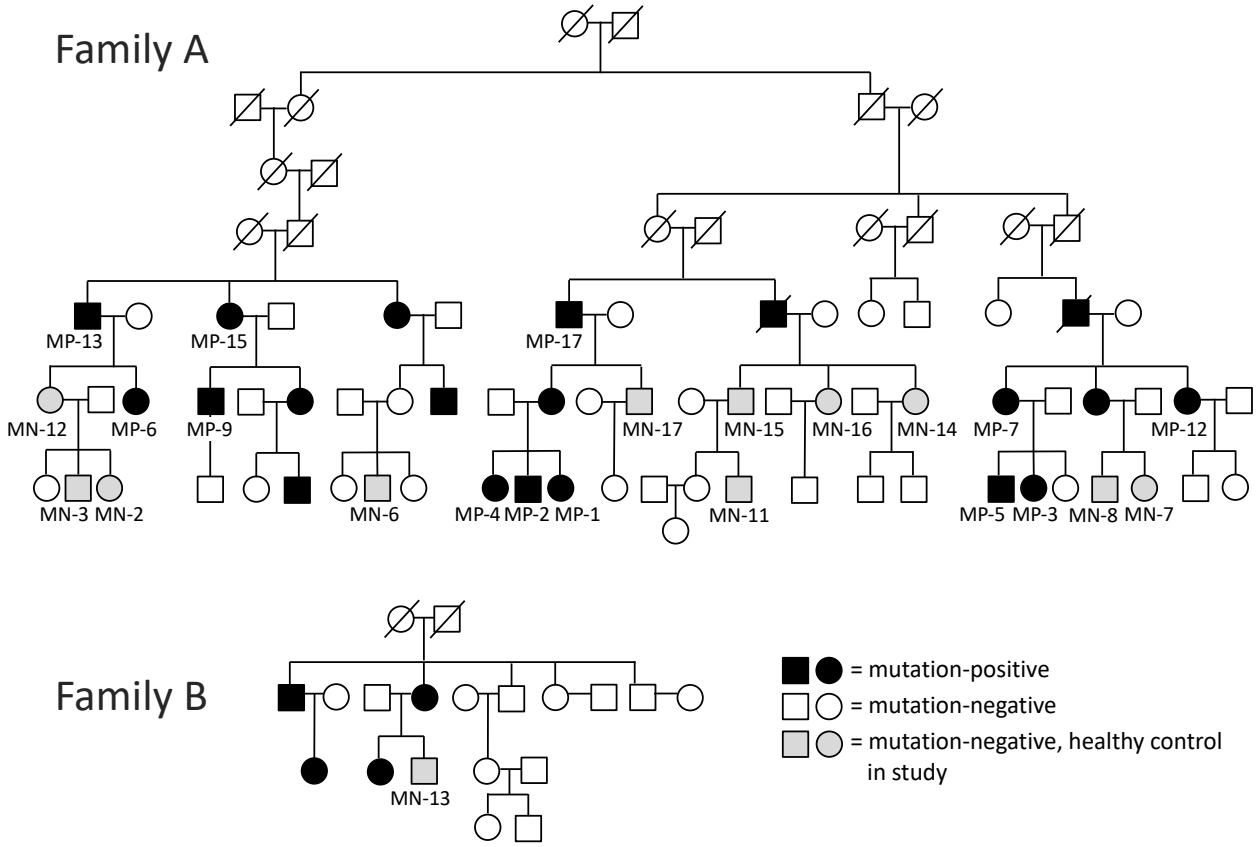


Figure 2. **A)** Hierarchical clustering and heatmap representation of 187 circulating microRNAs, with labelling of genotype, sex, and subfamily status in 12 mutation-positive subjects with a heterozygous missense mutation p.C218G in *WNT1*. Expression information of all 187 microRNAs was used as input for hierarchical clustering. Average linkage and correlation were used as distance metrics. **B)** Volcano plot highlighting circulating miRNA regulation in 12 mutation-positive subjects with a heterozygous missense mutation p.C218G in *WNT1*. For every analyzed miRNA (dots) the observed fold difference (log_2 transformed) between mutation positive and mutation negative subjects (x-axis) is shown in combination with the p-value derived from parametric t-statistics. MicroRNAs with $p<0.05$ are labelled.

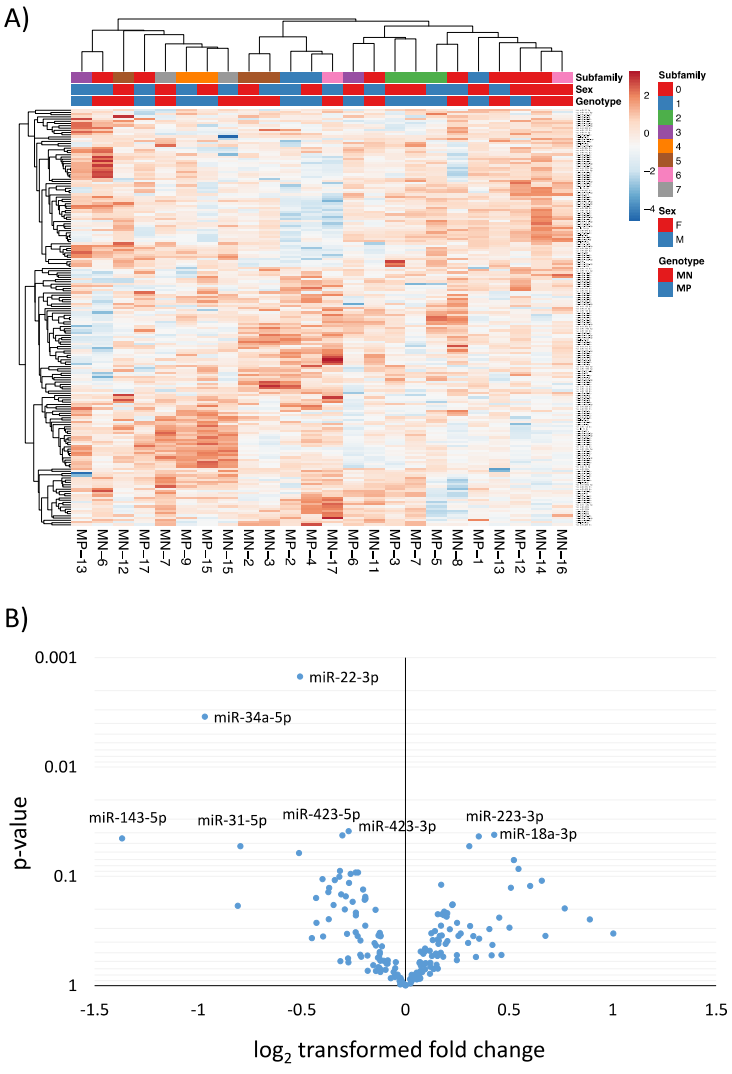
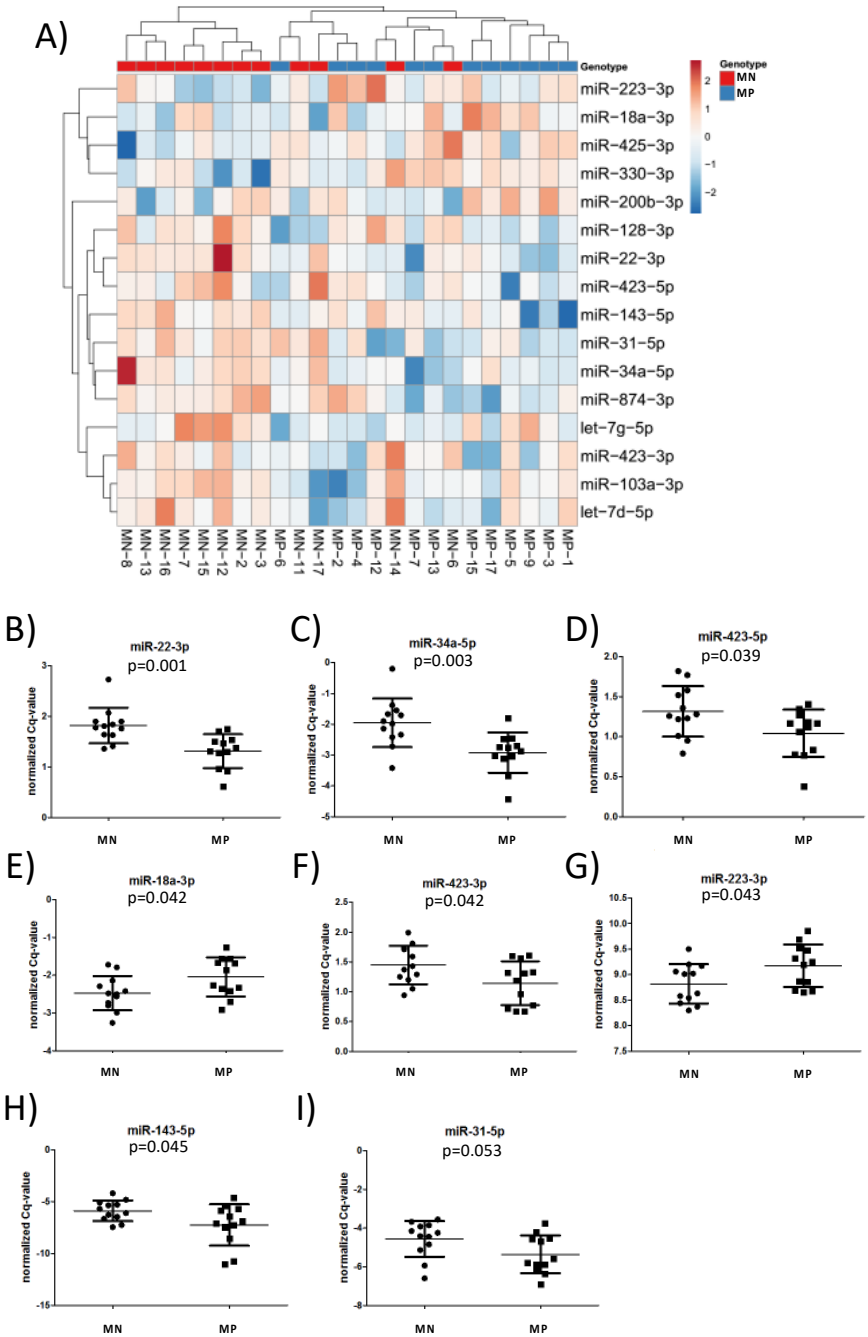


Figure 3. Heatmap with hierarchical clustering and scatterplots. **A)** The top 16 miRNAs (sorted according to p-value with p<0.1) in 12 mutation-positive subjects with a heterozygous missense mutation p.C218G in *WNT1* were used for hierarchical clustering. Average linkage and correlation were used as distance metrics. **B) – I)** Normalized (global mean) delta Cq-values are shown for seven significantly regulated miRNAs (p<0.05) and miR-31-5p (p=0.053). Parametric t-test was applied.



TABLES

Table 1. Biochemical findings in 12 MP subjects with the heterozygous *WNT1* mutation p.C218G and in 12 MN subjects

SUBJECT CODE	AGE (years)	SEX	S-Ca-ion (mmol/L)	P-Pi (mmol/L)	P-ALP (U/L)	S-D-25 (nmol/L)	S-D-1,25 (pmol/L)	S-FGF23, I (pg/mL)	S-iPTH (pg/mL)	S-CTX-1 (ng/mL)
Mutation-positive subjects (n = 12)										
MP-1	11	F	1.26	1.35	258	90	173.0	42.00	22.0	1.792
MP-2	13	M	1.25	1.66	247	112	94.3	77.27	**7.6**	2.946
MP-3	13	F	1.32	1.23	122	75	140.0	66.36	23.8	1.349
MP-4	17	F	1.25	1.12	77	116	96.4	75.45	31.4	0.562
MP-5	19	M	1.27	0.82	83	**31**	168.0	32.90	22.6	0.452
MP-6	34	F	1.24	1.25	84	105	**44.70**	63.77	40.0	0.147
MP-7	44	F	1.32	0.89	51	82	66.8	51.04	24.9	0.102
MP-9	52	M	1.24	0.86	50	74	172.0	54.68	24.3	0.196
MP-12	54	F	1.22	1.34	74	136	64.1	46.14	30.8	0.710
MP-13	63	M	1.24	1.09	52	146	114.0	66.10	29.3	0.107
MP-15	71	F	1.24	1.39	72	88	124.0	41.17	18.2	0.199
MP-17	76	M	1.28	0.85	84	89	87.6	54.94	47.9	0.161
Mutation-negative subjects (n = 12)										
MN-2	9	F	1.28	1.55	257	68	165.0	49.45	33.0	1.685
MN-3	10	M	1.32	1.42	313	70	192.0	37.03	12.6	1.636
MN-6	24	M	1.24	1.53	67	**39**	144.0	43.93	26.2	0.861
MN-7	27	F	1.27	1.38	60	70	127.0	51.56	36.6	0.731

MN-8	30	M	1.24	0.88	104	54	117.0	56.49	38.6	0.376
MN-11	32	M	1.27	**0.51**	67	**41**	92.8	65.84	29.8	0.332
MN-12	37	F	1.27	1.13	52	60	134.0	39.52	33.4	0.213
MN-13	43	M	1.22	0.84	61	68	74.1	74.16	35.6	0.284
MN-14	49	F	1.22	1.05	63	90	82.9	43.66	46.1	0.152
MN-15	53	M	1.28	1.09	50	73	57.2	61.17	56.4	0.268
MN-16	57	F	1.26	1.08	83	**39**	109.0	41.17	49.7	0.624
MN-17	59	M	1.28	1.02	94	**21**	71.1	77.79	35.8	0.223

Normal ranges according to HUSLAB Laboratory (females/males): S-Ca-ion: 1.16–1.3 mmol/L. P-Pi: 2–12 y, 1.2–1.8 mmol/L; 13–16 y, 1.1–1.8 mmol/L; 17 y, 0.8–1.4 mmol/L; females .18 y, 0.76–1.41 mmol/L; males 18–49 y, 0.71–1.53 mmol/L; males .50 y, 0.71–1.23 mmol/L. P-ALP: 8–9 y, 115–345 U/L; 10–11 y, 115–435/115–335 U/L; 12–13 y, 90–335/125–405 U/L; 14–15 y, 80–210/80–445 U/L; 16–18 y, 35–125/55–330 U/L; .18 y, 35–105 U/L. S-D- 25: .50 nmol/L. Normal range for D-1,25 (1,25-dihydroxyvitamin D) according to United Medix Laboratories Ltd: 48–190 pmol/L. Normal ranges for iFGF23 according to Immutopics International and Kainos Laboratories: 8.2–54.3 pg/mL. Normal ranges for iPTH and CTX according to IDS-iSYS Kit Manual (Immunodiagnostic Systems, Ltd., Bolton, UK): iPTH: adults, 11.5–78.4 pg/mL; CTX-1: premenopausal females, 0.034–0.635 ng/mL; post- menopausal females, 0.034–1.037 ng/mL; males, 0.038–0.724 ng/mL. Supranormal values are underlined, and subnormal values are in bold.

Abbreviations: CTX, collagen type 1 cross-linked C-telopeptide; F, female; iFGF23, intact fibroblast growth factor 23; iPTH, intact parathyroid hormone; M, male; P-ALP, alkaline phosphatase; P-Pi, phosphate; S-Ca-ion, calcium; S-D-25, 25-hydroxyvitamin D.

Table 2. Differential expression for putative microRNA biomarker candidates with p-value <0.1 in 12 MP subjects with the heterozygous missense mutation p.C218G in *WNT1* and in 12 MN subjects

miRNA-ID	**Average (normalized delta Cq)**		**Standard Deviation (Cq-value)**		**Fold Change (log_2-transformed)**	**Fold Change (linear)**	**Parametric t-Test**	**ROC Analysis**
Group	**MP**	**MN**	**MP**	**MN**	**MP vs. MN**	**MP vs. MN**	**p-value**	**AUC-value**
miR-22-3p	1.31	1.82	0.32	0.34	-0.51	0.70	**0.001**	0.896
miR-34a-5p	-2.92	-1.95	0.63	0.76	-0.97	0.51	**0.003**	0.868
miR-423-5p	1.04	1.32	0.28	0.3	-0.27	0.83	**0.039**	0.743
miR-18a-3p	-2.05	-2.47	0.49	0.43	0.43	1.35	**0.042**	0.757
miR-423-3p	1.14	1.45	0.35	0.31	-0.30	0.81	**0.042**	0.715
miR-223-3p	9.17	8.82	0.4	0.37	0.35	1.27	**0.043**	0.757
miR-143-5p	-7.24	-5.88	1.91	0.95	-1.37	0.39	**0.045**	0.726
miR-31-5p	-5.36	-4.56	0.94	0.89	-0.80	0.57	0.053	0.729
miR-425-3p	-0.96	-1.27	0.27	0.42	0.31	1.24	0.053	0.774
miR-874-3p	-2.19	-1.68	0.67	0.54	-0.51	0.70	0.061	0.736
miR-200b-3p	-2.87	-3.39	0.55	0.73	0.52	1.43	0.071	0.688
miR-330-3p	-4.01	-4.55	0.42	0.91	0.55	1.46	0.086	0.684
miR-103a-3p	4.69	5.01	0.39	0.44	-0.32	0.80	0.089	0.729
let-7g-5p	3.83	4.06	0.31	0.3	-0.23	0.85	0.092	0.722
miR-128-3p	-1.78	-1.54	0.33	0.31	-0.24	0.85	0.092	0.698
let-7d-5p	0.47	0.74	0.31	0.39	-0.26	0.84	0.095	0.705

miRNA/miR = microRNA, let = lethal-gene, Cq = quantification value, MP = mutation-positive, MN = mutation-negative, AUC = area-under-the-curve from ROC analysis. In bold, miRNAs that show p-values below 0.05.

Table 3. Previously reported data on the role in bone metabolism of the 7 discriminative miRNAs in *WNT1* MP subjects.

miRNA-ID	**Finding in this study**	**Role in bone metabolism**	**Target proteins in bone**	**References**
miR-22-3p	Downregulated	Negatively regulates osteogenesis and osteoblastogenesis	WNT1, Tcf7, Ep300	10,35
miR-34a-5p	Downregulated	Inhibits osteoblast differentiation and proliferation, increases osteoclast differentiation, elevates resorption, leads to decreased bone mineralization	JAG1, WNT1	7,36,53
miR-423-5p	Downregulated	Serum levels correlate negatively with fracture risk and bone quality	-	57
miR-18a-3p	Upregulated	Upregulated in osteosarcoma tissue	-	58
miR-223-3p	Upregulated	Regulates osteoclast differentiation, modulates expression of osteoclast marker genes NF-kB, TNF- α, and osteoprotegerin.	NFIA, FGFR2, IKKα,	59-62
miR-143-5p	Downregulated	Suppresses osteogenic differentiation, downregulated in osteosarcoma tissue	Osx	63,64
miR-31-5p	Downregulated	Inhibits osteogenesis and osteogenic differentiation of MSCs; increases osteoclastogenesis	FZD3, RhoA, SATB2, RUNX2, Osterix	8,9,65,66

miRNA/miR = microRNA.

Altered MicroRNA Profile in Osteoporosis Caused by Impaired WNT Signaling

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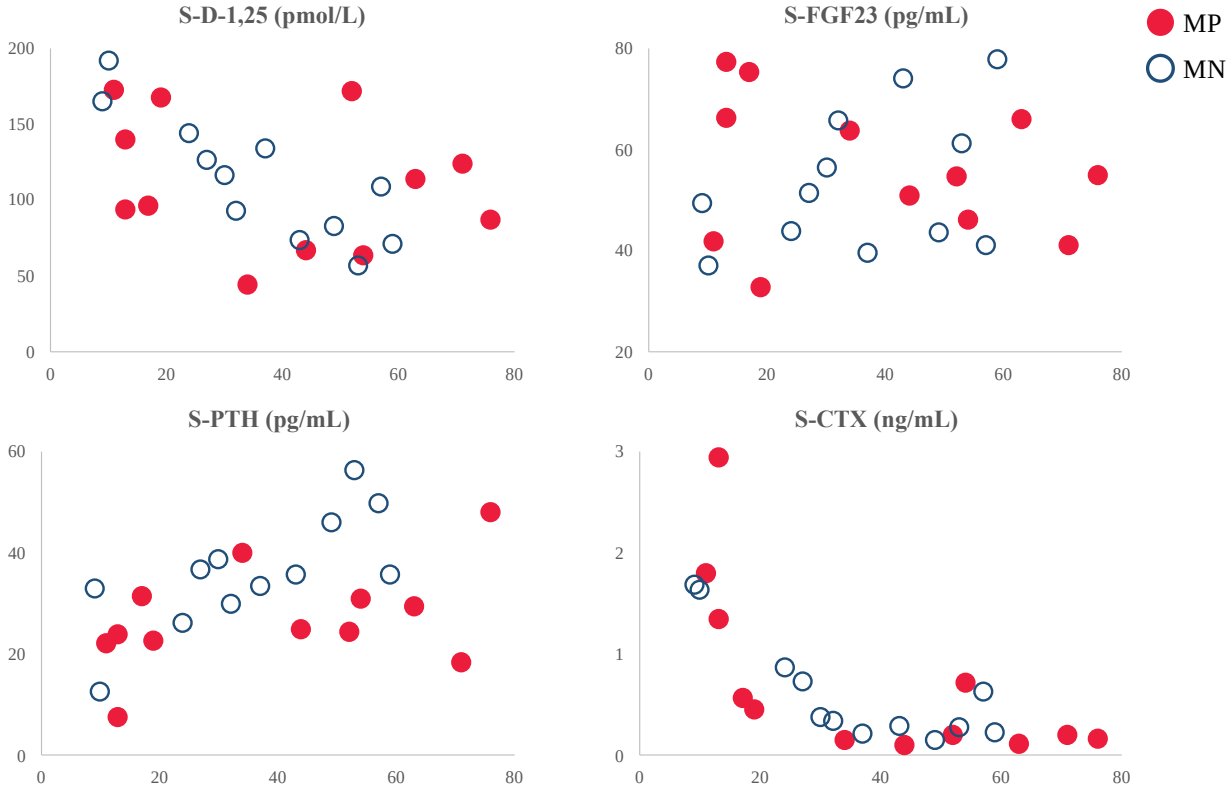
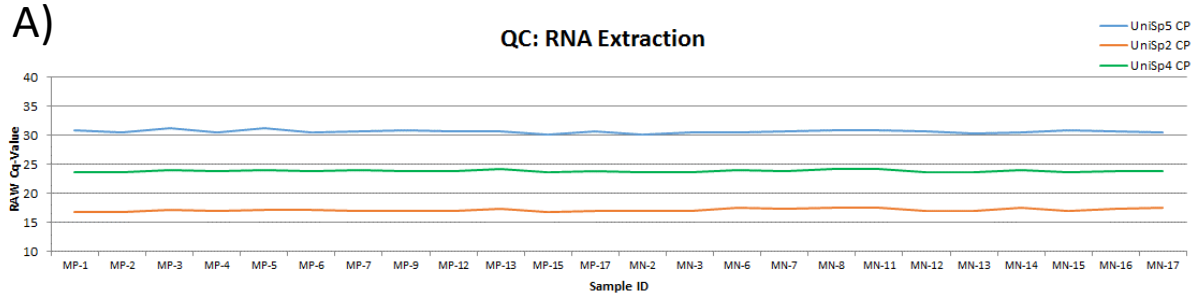
SUPPLEMENTAL DATA

Supplemental Figure 1. Serum concentrations of 1,25-dihydroxyvitamin D (S-D-1,25), intact fibroblast growth factor 23 (FGF23), parathyroid hormone (PTH), and collagen type 1 cross-linked C-telopeptide (CTX-1) in 12 mutation-positive subjects (MP) with a heterozygous missense mutation p.C218G in *WNT1* and in 12 mutation-negative subjects (MN).

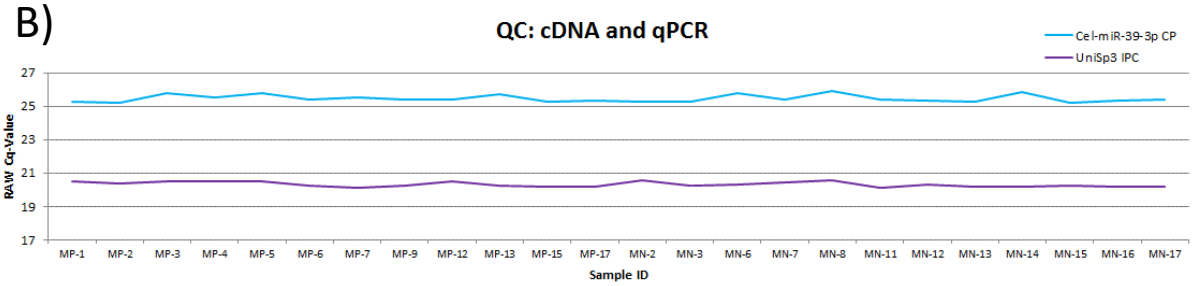


Supplemental Figure 2. Quality control for serum samples from 12 mutation-positive subjects with a heterozygous missense mutation p.C218G in *WNT1* and in 12 mutation-negative subjects. Raw (not normalized) Cq-values are shown across all 24 samples for A) RNA spike-in controls, and B) reverse transcription and qPCR spike-in controls. C) Table summarizing mean, median, standard deviation, and CV (%) for spike-in Cq-values. D) A ratio of >7 between miR-23a-3p and miR-451a is indicative of hemolysis (red line).

A)



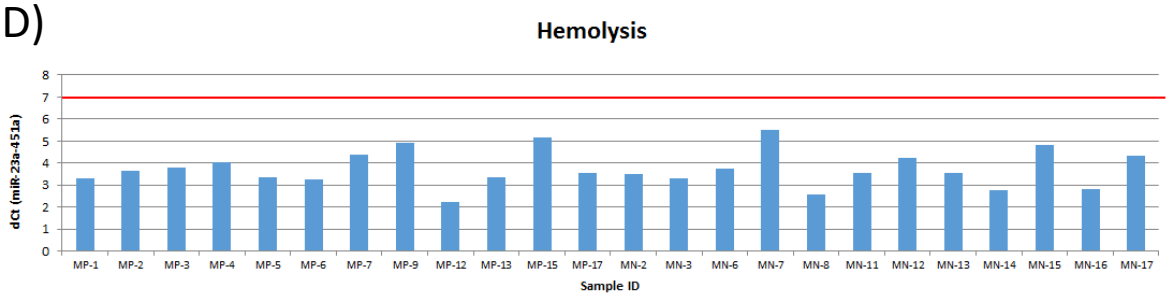
B)



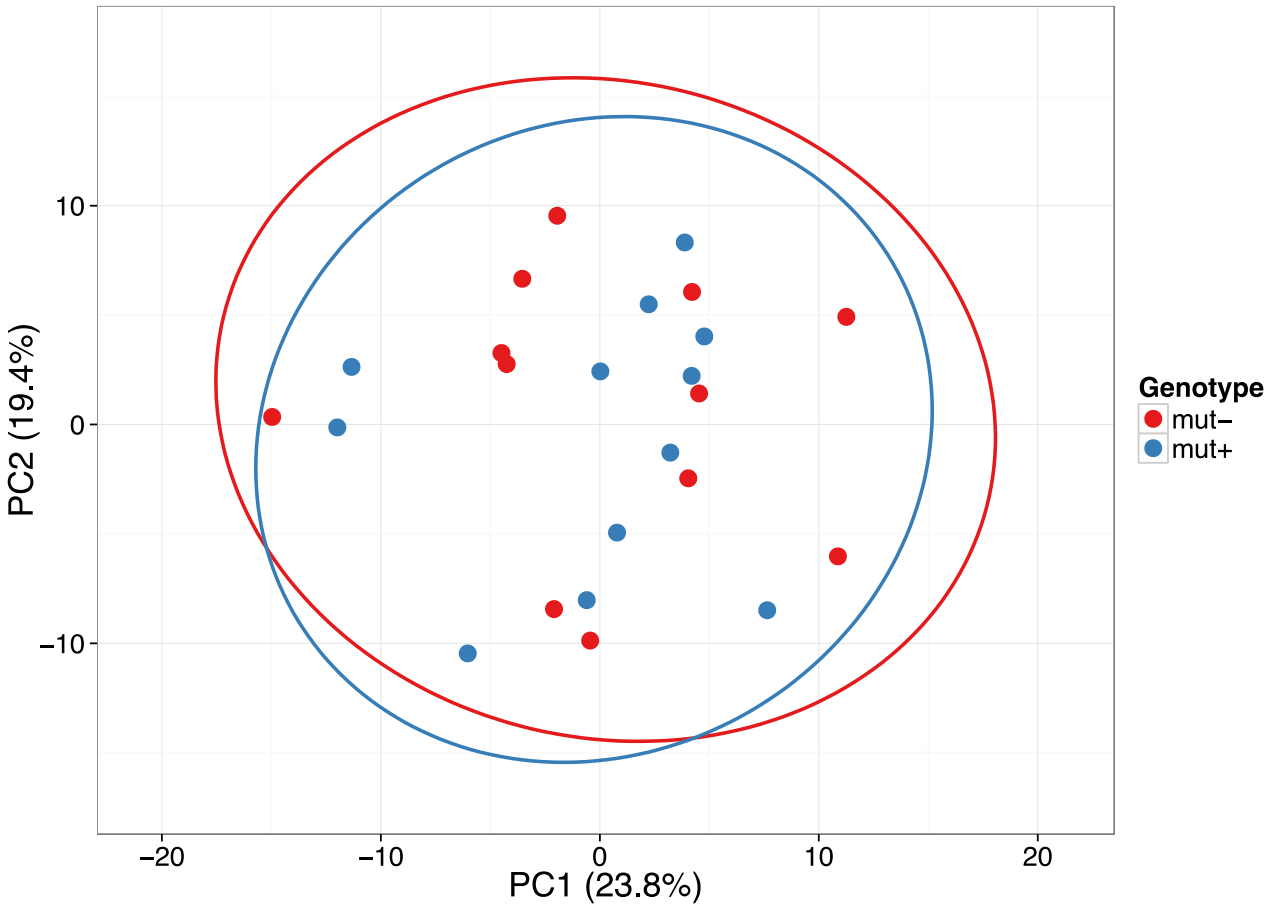
C)

Spike-In	Mean	Median	SD	CV
UniSp3 IPC	20.3	20.3	0.15	11%
Cel-miR-39-3p CP	25.5	25.4	0.22	16%
UniSp2 CP	17.1	17.0	0.24	18%
UniSp4 CP	23.9	23.8	0.18	13%
UniSp5 CP	30.7	30.7	0.30	23%

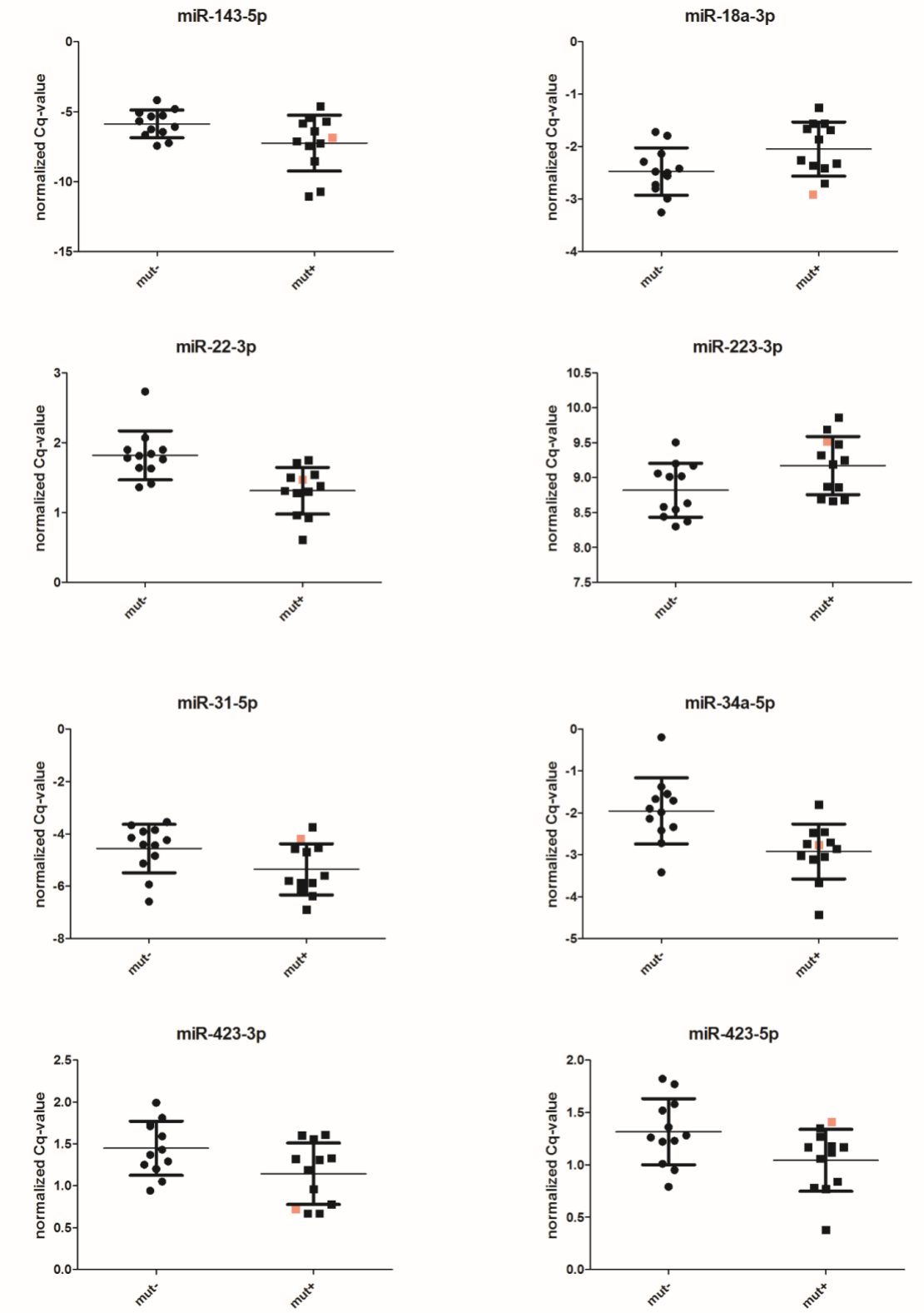
D)



Supplemental Figure 3. Exploratory data analysis of circulation miRNAs in 12 mutation-positive subjects with a heterozygous missense mutation p.C218G in *WNT1* and in 12 mutation-negative subjects. Principal component analysis was performed based on all 187 quantified microRNAs. Red dots represent samples from mutation-positive subjects, blue dots represent samples from mutation-negative subjects.



Supplemental Figure 4. Scatterplots for 8 miRNAs in 12 mutation-positive subjects with p.C218G in *WNT1* and in 12 mutation-negative subjects, and effect of bisphosphonate therapy on miRNAs. Normalized (global mean) delta Cq values are shown for 7 significantly regulated miRNAs ($p<0.05$) and miR-31-5p ($p=0.053$). One mutation-positive subject (MP-4) with on-going bisphosphonate treatment at the time of the study, is marked red.



Supplemental Table 1. Clinical findings in 12 mutation-positive subjects with a heterozygous *WNT1* mutation p.C218G and in 12 mutation-negative subjects.

SUBJECT CODE	AGE (years)	SEX	BMD Z-SCORES			FRACTURES		OSTEOPOROSIS MEDICATION
			LS	Fem	WB	Peripheral	Vertebral	
Mutation-positive subjects (n = 12)								
MP-1	11	F	-0.5	0.01	-0.5	1	0	-
MP-2	13	M	-1.1	-0.5	-0.7	4	0	-
MP-3	13	F	-2.1	-1.3	NA	1	0	-
MP-4	17	F	-2.5	-0.5	-1.0	9	0	PAM 2015-A
MP-5	19	M	-2.2	-0.1	NA	2	0	-
MP-6	34	F	-1.5	-2.2	-2.2	2	0	-
MP-7	44	F	-1.4	-0.9	NA	0	0	-
MP-9	52	M	-2.8	-1.3	-2.3	2	>3	-
MP-12	54	F	-3.3	-1.1	-1.5	0	0	-
MP-13	63	M	-2.2	-1.3	-3.5	3	>8	ZOL 2010, PTH 2013
MP-15	71	F	-1.5	-1.6	NA	9	>7	Estrogen N/A, RIS 2006
MP-17	76	M	0.05	0.01	NA	1	>5	ZOL 2008, PTH 2011-2012
Mutation-negative subjects (n = 12)								
MN-2	9	F	N/A	N/A	N/A	1	0	-
MN-3	10	M	N/A	N/A	N/A	0	0	-
MN-6	24	M	-0.2	-0.5	-0.5	0	0	-
MN-7	27	F	N/A	N/A	N/A	0	0	-
MN-8	30	M	N/A	N/A	N/A	0	0	-
MN-11	32	M	N/A	N/A	N/A	0	0	-
MN-12	37	F	-0.4	-0.2	-0.6	0	0	-

MN-13	43	M	N/A	N/A	N/A	0	N/A	-
MN-14	49	F	N/A	N/A	N/A	0	0	-
MN-15	53	M	N/A	N/A	N/A	0	1	-
MN-16	57	F	-1.2	-1.1	-1.1	0	0	-
MN-17	59	M	N/A	N/A	N/A	1	0	-

BMD = bone mineral density measured with dual-energy X-ray absorptiometry, LS = lumbar spine, FEM = femoral neck/femoral head for under-aged subjects, WB = whole body. PAM = pamidronate, ZOL = zsoledronic acid, PTH = teriparatide, RIS = risedronate. N/A = not available.
A Last dose given 4 months prior to obtaining samples.

Supplemental Table 2. Pearson correlation coefficients between significantly regulated miRNAs in 12 mutation-positive subjects with a heterozygous missense mutation p.C218G in *WNT1* and in 12 mutation-negative subjects are shown.

	miR-22-3p	*miR-34a-5p*	*miR-423-5p*	*miR-18a-3p*	*miR-423-3p*	*miR-223-3p*	*miR-143-5p*	*miR-31-5p*
miR-22-3p	1,00							
miR-34a-5p	0,63	1,00						
miR-423-5p	0,67	0,32	1,00					
miR-18a-3p	-0,27	-0,37	-0,15	1,00				
miR-423-3p	0,12	0,05	-0,14	-0,33	1,00			
miR-223-3p	-0,21	-0,15	-0,06	0,04	0,09	1,00		
miR-143-5p	0,45	0,38	0,09	-0,25	0,20	0,02	1,00	
miR-31-5p	0,40	0,46	0,11	-0,54	-0,13	-0,48	0,34	1,00